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Functional characterization of $P2Y_1$ and P2X4 receptors in human neuroblastoma SK-N-MC cells

Title in Spanish: *Caracterización funcional de receptores $P2Y_1$ y P2X4 en células SK-N-MC de neuroblastoma humano*

Javier Gualix[1*], Miriam León-Otegui[1], María Recuero[2], María Jesús Bullido[2], Fernando Valdivieso[2] and M. Teresa Miras-Portugal[1]

[1]Departamento de Bioquímica, Facultad de Veterinaria, Universidad Complutense de Madrid, 28040 Madrid, Spain. [2]Centro de Biología Molecular "Severo Ochoa" (C.S.I.C.-U.A.M.). Universidad Autónoma de Madrid, 28049, Madrid, Spain. Centro de Investigación Biomédica en Red sobre Enfermedades Neurodegenerativas (CIBERNED), Madrid, Spain. IdiPAZ - Instituto de Investigación Hospital Universitario La Paz, Madrid, Spain

ABSTRACT: Nucleotides are important signalling molecules in both the peripheral and central nervous system. However, the *in vitro* study of their receptors can be hampered by the heterogeneity of primary neuronal cultures. The use of clonal neuroblastoma cell lines allows to circumvent this difficulty, so these lines are often used as a model to analyze the properties, regulation and physiological role of nucleotide receptors in neural tissues. Expression studies indicated the presence of $P2Y_1$, $P2Y_6$, $P2Y_{11}$, $P2Y_{13}$, P2X1, P2X4, P2X5, P2X6 and P2X7 proteins in SK-N-MC cells. Functional analyses showed transient $[Ca^{2+}]_i$ increases upon application of ADP, 2-MeSADP or ADPβS. Responses to these agonists seem to be mediated by a $P2Y_1$ receptor, as demonstrated by the almost complete blockade exerted by the $P2Y_1$-selective antagonist MRS2179. ATP was also able to induce $[Ca^{2+}]_i$ increases in SK-N-MC cells. Responses to ATP were partially blocked by MRS2179 and the P2X antagonist TNP-ATP, thus suggesting that ATP can interact with two different P2 receptors: a $P2Y_1$ receptor, inhibited by MRS2179, and a TNP-ATP sensitive P2X receptor. To characterize the P2X receptor responsible for the MRS2179-resistant component of the ATP response, we analyze the effect of several P2X agonists on $[Ca^{2+}]_i$. Cells did not show responses to either α,β-meATP or BzATP, although $[Ca^{2+}]_i$ increases could be observed when cells were challenged with CTP. Both the response to CTP and the MRS2179-resistant component of ATP response were potentiated by ivermectin. Such pharmacological profile is consistent with the presence of a functional $P2X_4$ receptor in SK-N-MC cell line.

RESUMEN: Los nucleótidos son importantes moléculas señalizadoras en el sistema nervioso. El estudio *in vitro* de sus receptores puede verse obstaculizado por la heterogeneidad de los cultivos neuronales. El uso de líneas celulares de neuroblastoma permite eludir esta dificultad y dichas líneas se utilizan frecuentemente como un modelo con el que analizar las propiedades, regulación y función de los receptores de nucleótidos en tejidos neurales. Estudios de expresión indicaron la presencia de proteínas $P2Y_1$, $P2Y_6$, $P2Y_{11}$, $P2Y_{13}$, P2X1, P2X4, P2X5, P2X6 y P2X7 en las células SK-N-MC. Análisis funcionales mostraron incrementos transitorios de $[Ca^{2+}]_i$ tras la aplicación de ADP, 2-MeSADP o ADPβS, respuestas que parecen estar mediadas a través un receptor $P2Y_1$, como se pone de manifiesto por el bloqueo casi total ejercido por el antagonista selectivo $P2Y_1$, MRS2179. El ATP también indujo incrementos de $[Ca^{2+}]_i$ en las células SK-N-MC, siendo su respuesta parcialmente bloqueada por MRS2179 y por el antagonista P2X TNP-ATP, lo que sugiere que el ATP puede interactuar con dos receptores P2 diferentes: un receptor $P2Y_1$, inhibido por MRS2179, y un receptor P2X sensible a TNP-ATP. Se caracterizó el receptor P2X analizando el efecto de varios agonistas en la $[Ca^{2+}]_i$. Ninguna célula mostró respuestas a α,β-meATP o BzATP, aunque se observaron incrementos de $[Ca^{2+}]_i$ cuando las células fueron estimuladas con CTP. Tanto la respuesta a CTP como el componente de la respuesta a ATP resistente a MRS2179, se potenciaron en presencia de ivermectina. Todos estos datos sugieren la presencia de un receptor P2X4 funcional en las células SK-N-MC.

[*]**Corresponding Author:** jgualix@ucm.es
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1. INTRODUCTION

Extracellular nucleotides have major roles in the activity of neural cells. They act as transmitters or modulators in most nerve types in the peripheral and central nervous system. Furthermore, they also act as important mediators in neuronal-glia or glia-glia communication and are trophic factors participating in cell differentiation, proliferation, migration and death (1, 2). Nucleotide-mediated signaling mechanisms and specific receptor subtypes have been shown to be involved in various pathological conditions including brain trauma and ischaemia, neurodegenerative diseases involving neuroimmune and neuroinflammatory reactions, as well as in neuropsychiatric diseases (3).

Nucleotide effects are mediated trough two subclasses of specific cell surface receptors, ionotropic P2X and metabotropic P2Y receptors (4). Seven distinct P2X subtypes (P2X1-7) have been cloned from mammalian species, showing a topology that includes two transmembrane spanning regions, a large extracellular loop and intracellular N and C termini. These proteins assemble into ATP activated channels either as homomers or heteromers, each functional receptor containing at least three monomers. P2X receptor activation results in Na^+ and Ca^{2+} influx across the cell membrane, which leads to depolarization. Membrane depolarization subsequently activates voltage-operated calcium channels, thus causing accumulation of Ca^{2+} ions in the cytoplasm. Although the detailed signalling mechanism have not been established for most P2X receptor subtypes, it is well known that cytoplasmic Ca^{2+} elevation triggers a variety of intracellular events, in part, trough activation of mitogen-activated protein kinases (MAPKs), protein kinase C (PKC) and calmodulin (5-7).

The P2Y receptors are classical 7-transmembrane domain metabotropic receptors coupled to G proteins. Currently, eight P2Y receptors subtypes ($P2Y_{1, 2, 4, 6, 11, 12, 13, 14}$) have been cloned. $P2Y_{1, 11, 12, 13}$ receptors are selectively activated by adenine nucleotides, whereas $P2Y_4$ and $P2Y_6$ are stimulated by pyrimidines. The $P2Y_2$ receptor responds equally well to purine and pyrimidine triphosphates (ATP and UTP) and $P2Y_{14}$ is a sugar-nucleotide (UDP-glucose) responding receptor. Most members of the P2Y family ($P2Y_{1, 2, 4, 6, 11}$) are functionally coupled to $G_{q/11}$ proteins and stimulate phosphoinositide-specific phospholipase C, resulting in the formation of inositol-(1,4,5)-trisphosphate and diacylglycerol with subsequent mobilization of Ca^{2+} from internal stores. $P2Y_{11}$ receptor additionally stimulates adenylate cyclase, whereas $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$ promote $G_{i/0}$ inhibition of adenylate cyclase activity. The stimulation of several P2Y receptors is also commonly associated with the activation of MAPKs (in particular extracellular signal-regulated protein kinase 1/2, ERK1/2). Other classes of protein, such as phospholipases A_2 and D, PKC or phosphatidylinositol 3-kinase, can be also activated according to the cell context and the particular P2Y subtype (5, 8, 9).

Several neuroblastoma cell lines, each originating from a single cell that has been isolated from a spontaneously occurring or induced tumor of the nervous system, are now available and can be maintained in culture infinitely. Neuroblastoma derived cell lines have been used as an *in vitro* model for the study of a variety of neurotransmitter receptors including those for nucleotides. Indeed, the $P2Y_2$ receptor was originally cloned from the mouse neuroblastoma/rat glioma hybrid line NG108-15 (10). Further studies have revealed the presence in these cells of the $P2X_7$ receptor and metabotropic receptors other than the $P2Y_2$ subtype (11, 12). Functional $P2X_7$ receptors can be also detected in different human neuroblastoma lines (13, 14) as well as in the mouse Neuro-2a neuroblastoma cells (6, 15). In addition, a range of cell lines of both mouse and human neuroblastoma origin have been shown to functionally express pyrimidine selective P2Y receptors (16-18).

SK-N-MC human neuroblastoma are known to constitutively express adrenergic α_{2C}, β_1 and β_3 (19, 20), dopamine D1 (21), neuropeptide Y_1 (22) and muscarinic M_1 receptors (23). The regulation and signalling pathways activated by these receptors have been extensively investigated using SK-N-MC cells as a model (24-28). SK-N-MC cell line also served to analyze the integration or cross-communication of signals upon activation of different types of neurotransmitter receptors (29). More recently, SK-N-MC has been used as a parental line with which generate cellular lines that stably overexpress proteins of interest in the study of distinct neurodegenerative disorders, such as the amyloid precursor protein APP (30), carboxyl-terminal fragments of the APP protein (31, 32), or different mutated forms of the Parkin protein (33).

Earlier experiments showed that ATP and other nucleotides are able to induce response (phosphoinositide hydrolysis and formation of inositol phosphates) in the SK-N-MC cells, thus indicating the presence of not fully characterized P2 receptors in this neuroblastoma cell line (23).

In the present study we have analyzed the presence of functional P2 receptors in the human neuroblastoma SK-N-MC cells. If such receptors were present in these cells, SK-N-MC cell line could serve as a useful model to analyze several aspects of the signalling mediated through nucleotide receptors in neural tissues. This includes the regulation and transduction pathways activated by P2 receptors, their interaction with other neurotransmitter receptors or their putative relationship with proteins involved in neurodegenerative processes

2. MATERIALS AND METHODS

2.1. Materials

2-methylthyo-adenosine-5'-diphosphate (2-MeSADP), α,β-methylene-adenosine 5'-triphosphate (α,β-meATP), ADP, adenosine-5'-0-(2-thiodiphosphate) (ADPβS), ATP, 2'(3')-O-(4-benzoylbenzoyl) adenosine 5'-triphosphate (BzATP), CTP, pyridoxalphosphate-6-azophenyl-2',4'-disulphonic acid (PPADS), 2',3'-*O*-(2,4,6-trinitrophenyl)adenosine 5'-triphosphate (TNP-ATP), UDP, UTP and ivermectin were purchased from Sigma (St Louis, Missouri, USA). N^6-methyl-2'-deoxyadenosine-3',5'-bisphosphate (MRS2179) was purchased from Tocris Cookson (Bristol, UK). Products for cell culture, immunocytochemical and western-blot studies and fluorescent probes are included in the specific methods section. Other analytical grade reagents were purchased from Merck (Darmstadt, Germany).

2.2. Cell culture

The SK-N-MC human neuroblastoma cell line (HTB-10) was purchased from the American Type Culture

Collection (ATCC, Barcelona, Spain). Cells were grown in monolayer in 10-cm plastic culture dishes (BD Falcon, Erembodegem, Belgium) at 37 ºC in a humidified atmosphere of 95% air and 5% CO_2. Culture medium was minimum essential medium (Gibco, Rockville, MD, USA) supplemented with 10% heat-inactivated fetal calf serum (Gibco), 2 mM glutamine, 1 mM sodium pyruvate, non-essential amino acids (alanine, glutamic acid, aspartic acid, proline and asparagine, each at 0.4 mM), 100 units/mL penicillin, 0.1 mg/ml streptomycin and 0.25 µg/mL amphotericin B (Sigma).

Cells were subcultured about 2 times a week: culture medium was removed and discarded and the cell layer was rinsed two times with 2 mL of a solution containing 0.25% (w/v) trypsin and 0,02% (w/v) EDTA in Hank's Balanced Salt Solution with phenol red (Sigma). Then, culture dishes were placed in an incubator for 1 min at 37º to allow cells to detach. After that, 5 mL of the culture medium were added to the dishes and cells were aspirated by gently pippeting. Cells were counted in a Neubauer chamber using the trypan blue exclusion test and appropriate aliquots of the cell suspension were taken for the immunocitochemical, western blot or calcium imaging assays. Finally, 1 mL of the cell suspension was added to a new culture dish containing 9 mL of the culture medium and cells were maintained at 37°C in 5% CO_2.

2.3. Immunocytochemical assays

Cells were plated at a density of 200,000 cells/mL on glass coverslips precoated with 0.1 mg/mL poly-L-lysine (Biochrom AG, Berlin Germany) and maintained in a humidified incubator at 37 ºC in 5% CO_2 for 2-3 days before they were used in the immunocytochemical experiments. Cover slips with the SK-N-MC cells attached were then treated with 4% (w/v) *p*-formaldehyde (Sigma) for 15 min, washed twice with phosphate-buffered saline (PBS) (composition mM: NaCl 137, KCl 2.6, KH_2PO_4 1.5, Na_2HPO_4 8.1, pH 7.4) and incubated for 1 h in PBS containing 3% (w/v) bovine serum albumine (BSA), 0.1% (v/v) Triton X-100 and 5% (v/v) normal goat or donkey serum (Sigma). Cells were washed three times with PBS/BSA and incubated with the primary antibody for 1 h at 37º C. Primary antibodies assayed were: rabbit anti-$P2Y_1$ (serum at 1:100 dilution) (Alomone labs, Jerusalem, Israel), rabbit anti-$P2Y_2$ (1:200) (Chemicon International, Temecula, CA, USA), rabbit anti-$P2Y_4$ (1:100) (Chemicon International), rabbit anti-$P2Y_6$ (1:100) (Alomone labs), rabbit anti-$P2Y_{11}$ (1:100) (Alomone labs), rabbit anti-$P2Y_{12}$ (1:100) (Alomone labs), rabbit anti $P2Y_{13}$ (1:50) (Alomone labs), rabbit anti-$P2X_1$ (1:100) (Chemicon International), guinea-pig anti-$P2X_2$ (1:250) (Chemicon International), guinea-pig anti-$P2X_3$ (1:500) (Chemicon International), rabbit anti-$P2X_4$ (1:100) (Alomone labs), rabbit anti-$P2X_5$ (1:200) (Alomone labs), rabbit anti-$P2X_6$ (1:100) (Alomone labs) or rabbit anti-$P2X_7$ (intracellular epitope, 1:100 dilution) (Alomone labs). All the antibodies used in this work were polyclonal and most of them were raised against the corresponding human protein. In some cases, antibodies against the rat protein were also used (if it was the case, cross reactivity with the human protein has been verified by the manufacturer). Next, covers were washed three times with PBS/BSA and incubated for 1 h at 37ºC with the secondary antibodies: donkey anti-rabbit IgG coupled to Cy3 (1:400) (Jackson Immunoresearch, West Grove, PA, USA) or fluorescein conjugated goat anti guinea-pig IgG (1:64) (Sigma). Then, covers were washed three times with PBS and mounted following standard procedures. Control experiments of nonspecific staining were performed without primary antibody or by preadsorption of the primary antibody with the immunizing peptide.

Fluorescent images were taken using a NIKON TE-200 microscope equipped with a S Fluor 40X objective (oil, 1.3 NA) a mercury lamp light source, fluorescein and rhodamine Nikon filter sets and a CCD camera (Kappa ACC 1) controlled by Kappa ImageBase software (Kappa opto-electronics, Gleichen, Germany).

2.4. Western blot

Cells were seeded on 10-cm plastic Petri dishes (BD Falcon) at a density of 400,000 cells/mL and maintained at 37 ºC in 5% CO_2 for 2-3 days before any treatment. Western blot analysis were performed using protein extracted from the SK-N-MC cells by homogenization in a buffer containing 100 nM HEPES (pH 7.4), 0.5 mM EDTA, 50 µg/ml phenylmethylsulfonyl fluoride, 0.5 µg/ml leupeptine, 0.5 µg/ml pepstatine, 0.2 M NaCl and 1% (v/v) Triton X-100. The samples were homogenized at 4°C and protein content determined by Bradford assay. Total protein (40 µg) was electrophoresed on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Whatman, Maidestone, UK). The membranes were blocked with 5% nonfat dried milk for 1 h at room temperature and further incubated overnight at 4ºC with the different P2 receptor antibodies at a dilution of 1:500. Blots were then washed and incubated for 1 h at room temperature with the corresponding secondary antibody coupled to horseradish peroxidase (DakoCytomation, Glostrup, Denmark). Protein bands were detected with enhanced chemoluminiscence detection (Perkin Elmer, Boston, MA, USA). Incubation with the control peptide was made following the manufacturer instructions.

2.5. Calcium imaging

Cells were plated at a density of 200,000 cells/mL on glass coverslips precoated with 0.1 mg/mL poly-L-lysine (Biochrom) and maintained in a humidified incubator at 37 ºC in 5% CO_2 for 2-3 days before they were used in the calcium imaging experiments. Cells attached to coverslips were then washed with Locke's solution (composition in mM: NaCl, 140; KCl, 4.7; MgSO4, 1.2; KH_2PO_4, 1.2; $CaCl_2$, 2.5; glucose, 5.5; HEPES, 10; pH 7.4) supplemented with 1 mg/mL BSA and loaded with 5 µM Fura-2 AM (Molecular Probes, Eugene, OR, USA) for 30 min at 37 ºC. Next, the coverslips were washed with Locke's medium and mounted in a small superfusion

chamber on the stage of a Nikon TE-200 microscope. Cells were superfused with Locke's solution, and different agonists of the P2 receptors were applied in 30s pulses. When the experiments were performed using P2 receptor antagonists (MRS2179, TNP-ATP) or potentiators (ivermectin), these compounds were superfused for 2 min before the application of the corresponding agonist.

Cells were alternately excited at 340 and 380 nm, these wavelengths corresponding to the fluorescence peaks of Ca^{2+}-saturated and Ca^{2+}-free Fura-2 solutions. The wavelength of the incoming light was selected with the aid of a Lambda 10-2 optical filter changer (Sutter Instrument, Novato, CA, USA) and emitted light was isolated with a dichroic mirror (430 nm) and a 510 nm bandpass filter (Omega Optical). Cells were imaged through a NIKON 40X lens (S Fluor 1.3 oil iris) and 12-bit images were acquired using an ORCA-ER C 47 42-80 camera from Hamamatsu (Hamamatsu City, Japan) controlled by MetaFluor 6.2r6 PC software (Universal Imaging Corp., Cambridge, UK). Time course data represent the average light intensity in a small elliptical region inside each cell. Background and autofluorescence components were subtracted at each wavelength and the 340/380 ratio was calculated. The data are represented as the normalized F340/F380 fluorescence ratio, which increases as $[Ca^{2+}]_i$ increases.

3. RESULTS

3.1. Immunological characterization of P2X subunits in SK-N-MC cells

In order to investigate the presence of native purinergic receptors in the SK-N-MC cells, the expression of P2X subunits was analyzed by using commercially available subunit-specific antibodies.

Western blot experiments demonstrate that most of the P2X subunits are expressed in SK-N-MC cells, as bands corresponding to monomeric P2X1, P2X4, P2X5, P2X6 and P2X7 proteins can be immunodetected (results not shown).

As expected, immunocytochemical detection of P2X subunits correlated well with the results obtained by western blot. All P2X subunit antibodies, except that for the P2X2 and P2X3 subtypes, labeled the SK-N-MC cells. Immunostaining was specific as it disappeared when antibodies were pre-adsorbed with the corresponding antigen peptide (Figure 1).

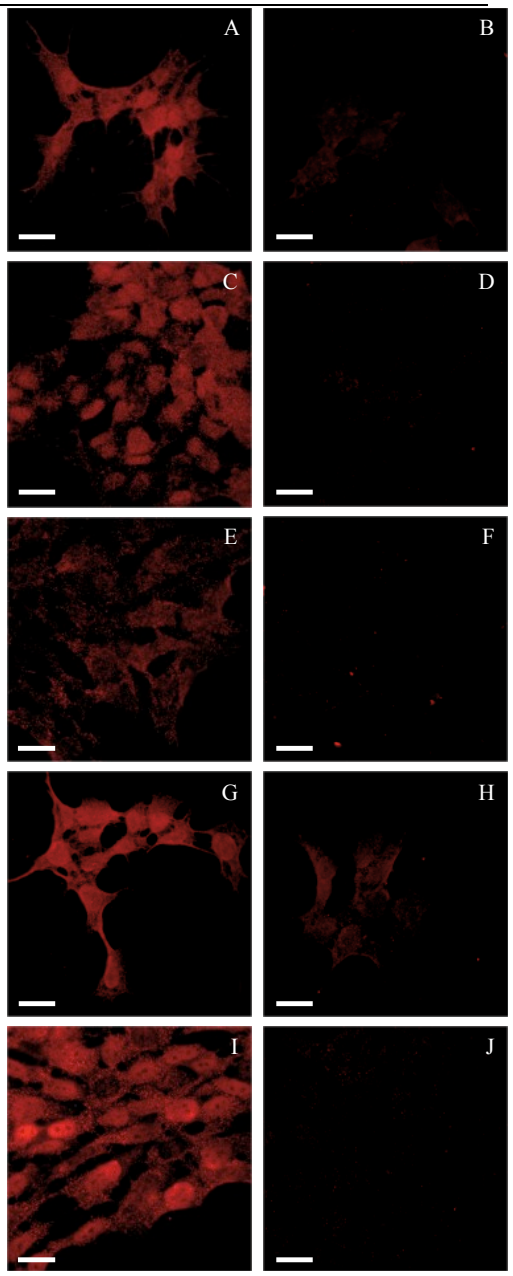

Figure 1. Immunological detection os P2X receptor subunits in SK-N-MC cells. Fluorescence image of SK-N-MC cells labeled with anti-$P2X_1$ (**A**), anti-$P2X_4$ (**C**), anti-$P2X_5$ (**E**), anti-$P2X_6$ (**G**) and anti-$P2X_7$ (**I**) antibodies. To confirm a specific immunoreaction, primary antibodies used in **A**, **C**, **E**, **G** and **I** were pre-absorbed with the corresponding control peptide and immunostaining are shown in **B**, **D**, **F**, **H** and **J**, respectively. Scale bar= 20 μm in all micrographs.

3.2. Immunological characterization of P2Y receptors in SK-N-MC cells

Immunological characterization of P2Y receptors was carried out using commercially available antibodies for the $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$ and $P2Y_{13}$ subtypes.

Several P2Y receptors are simultaneously expressed in SK-N-MC cells, as specific bands corresponding to $P2Y_1$,

$P2Y_6$, $P2Y_{11}$ and $P2Y_{13}$ proteins can be detected in the western blot assays (results not shown). Again, immunocytochemical experiments correlated with the results obtained in the western blot experiments: SK-N-MC cells showed specific inmmunostaining with antibodies against $P2Y_1$, $P2Y_6$, $P2Y_{11}$ and $P2Y_{13}$ receptor subtypes (Figure 2). No immunostaining were observed with the antibodies for the $P2Y_2$, $P2Y_4$ and $P2Y_{12}$ subtypes (results not shown).

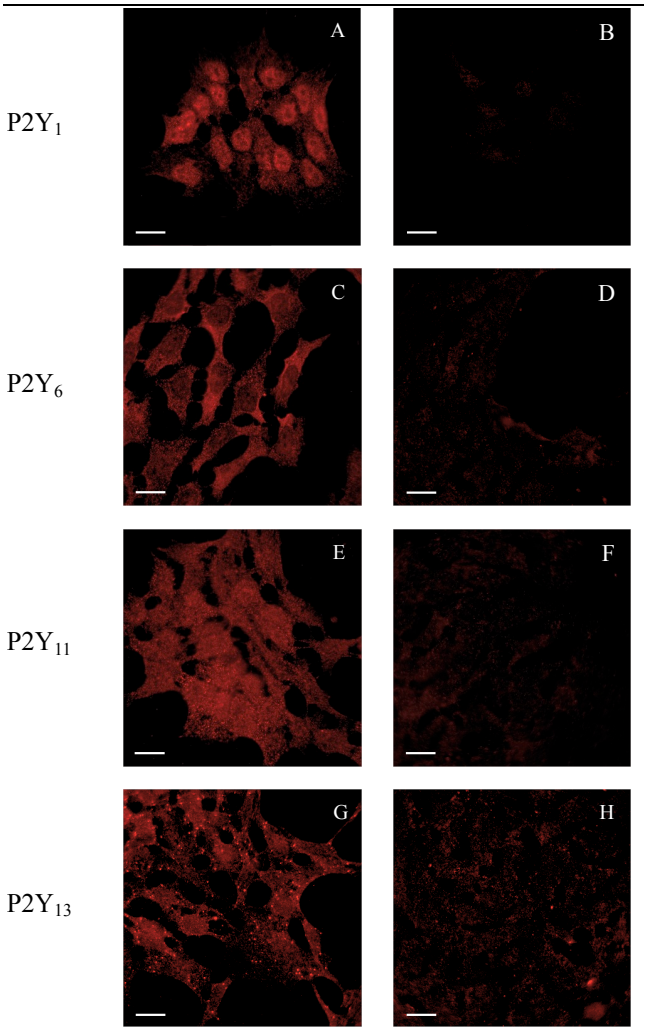

Figure 2. P2Y receptors immunodetection in SK-N-MC cells. Fluorescence image of SK-N-MC cells labeled with anti-$P2Y_6$ (**C**), anti-$P2Y_{11}$ (**E**) and anti-$P2Y_{13}$ (**G**) antibodies. To confirm a specific immunoreaction, the primary antibodies used in **A**, **C**, **E** and **G** were pre-absorbed with their corresponding control peptide and immunostaining are shown in **B**, **D**, **F** and **H**, respectively. Scale bar= 20 µm in all micrographs.

3.3. Characterization of functional P2 receptors by calcium imaging in SK-N-MC cells

The immunodetection of different P2X subunits or P2Y receptors indicates the presence of the protein but does not give information concerning their functionality. To approach this specific issue, microfluorimetric techniques were used.

We analyzed the response of SK-N-MC cells to the most commonly utilized P2 agonists by single-cell imaging after loading cells with the fluorescent calcium dye Fura-2 AM. A significant percentage of the cells (91.5 ± 1.5% of total cells, n = 240) showed transient $[Ca^{2+}]_i$ increases upon application of 10 µM ADP. Response to ADP is likely mediated by a $P2Y_1$ receptor, as demonstrated by the almost complete blockade exerted by the $P2Y_1$-selective antagonist MRS2179 (10 µM, Fig. 3A). Other $P2Y_1$ agonists such as 2-MeSADP (10 µM) or ADPβS (10 µM) were also able to elicit $[Ca^{2+}]_i$ increases in the SK-N-MC cells, these responses being almost completely blocked by 10 µM MRS2179 (Fig. 3B,C). Neither UDP (100 µM) nor UTP (100 µM) elicited calcium responses in the SK-N-MC cells (results not shown), thus precluding the presence of functional pyrimidine receptors.

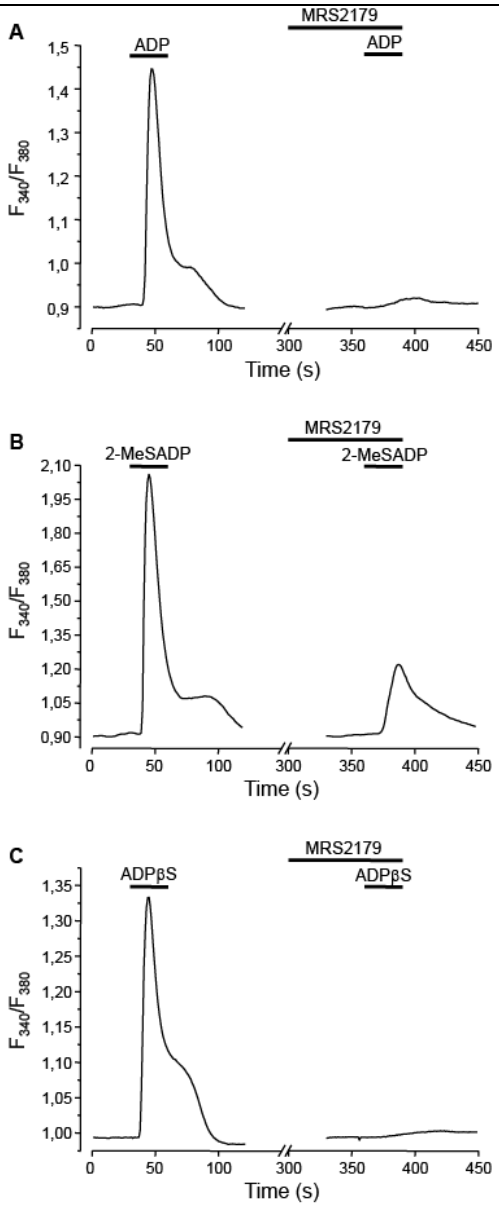

Figure 3. Stimulation of the SK-N-MC cells with ADP, 2-MeSADP or ADPβS induces intracelular transients that are inhibited by the $P2Y_1$ antagonist MRS2179. Changes in the

intracelular Ca^{2+} concentration were measured when cells were challenged with 10μM ADP (**A**), 10 μM 2-MeSADP (**B**) OR 10 μM AdβS (**C**), both in the absence and in the presence of the selective $P2Y_1$ antagonist, MRS 2179 (10 μM). Traces represent the mean of the responses measured in 156 (**A**), 140 (**B**) or 144 (**C**) individual cells. Horizontal bars above traces indicate duration of drug applications.

We also tested the ability of ATP (100 μM) to induce $[Ca^{2+}]_i$ increases in the SK-N-MC cells. Almost all SK-N-MC cells (96.8 ± 0.4% of total cells, *n* = 882) showed responses when challenged with this nucleotide. Thus, ATP was used in some of our assays as an indicator of cell functionality (see Figures 5A and B). Responses to 100 μM ATP were partially blocked by MRS2179 (10 μM), the residual response observed in the presence of the antagonist being a 13.3 % of the original one (Fig 4A). The P2X antagonist TNP-ATP (100 μM) also acted as a partial inhibitor of ATP responses, reducing by 50.2 % the amplitude of the calcium transients elicited by the agonist (Fig 4B). When SK-N-MC cells were treated with both antagonists, MRS2179 (10 μM) and TNP-ATP (100 μM), responses to 100 μM ATP were completely blocked (results not shown). This is indicating that ATP is exerting its effects through the interaction with two different P2 receptors: a $P2Y_1$ receptor, sensitive to MRS2179, and a P2X receptor which is blocked by TNP-ATP.

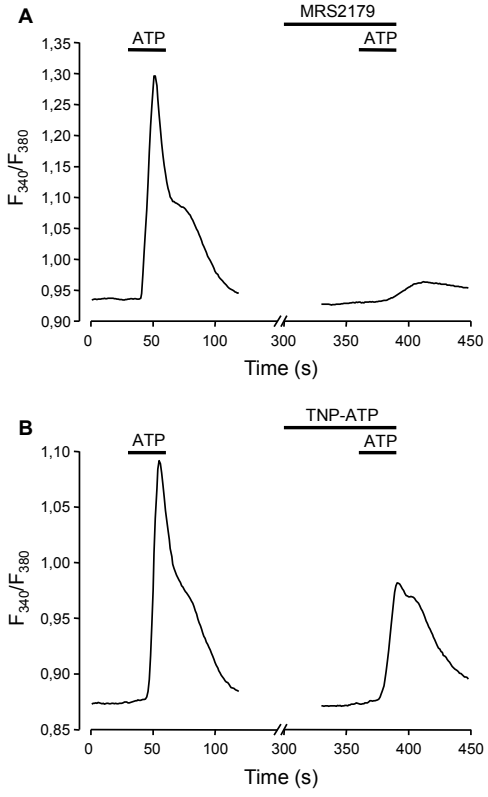

Figure 4. ATP induces calcium responses in the SK-N-MC cells that are inhibited by MRS2179 and TNP-ATP. Changes in the intracellular Ca^{2+} concentration were measured when cells were stimulated with 100 μM ATP applied alone or in the presence of the selective $P2Y_1$ antagonist, MRS 2179 (10 μM, **A**), or the P2X antagonist, TNP-ATP (100 μM, **B**). Traces represent the mean of the responses obtained in 128 (**A**) or 94 (**B**) individual cells. Horizontal bars above traces indicate duration of drug applications.

To further characterize the P2X receptor that is responsible for the MRS2179 resistant component of the ATP response, we analyze the effect of several P2X agonists on the SK-N-MC cells. No cells showed responses to either 100 μM α,β-meATP or 100 μM BzATP (Fig 5A). However, responses to CTP (300 μM) were present in a 67.6 ± 10.1% of the analysed cells (*n* = 396, Fig 5B). Moreover, both the response to CTP (300 μM) and the MRS2179-resistant component of the ATP (100 μM) response were potentiated in the presence of 5 μM ivermectin (Fig 5B and C), a positive allosteric modulator of the P2X4 receptor (34, 35).

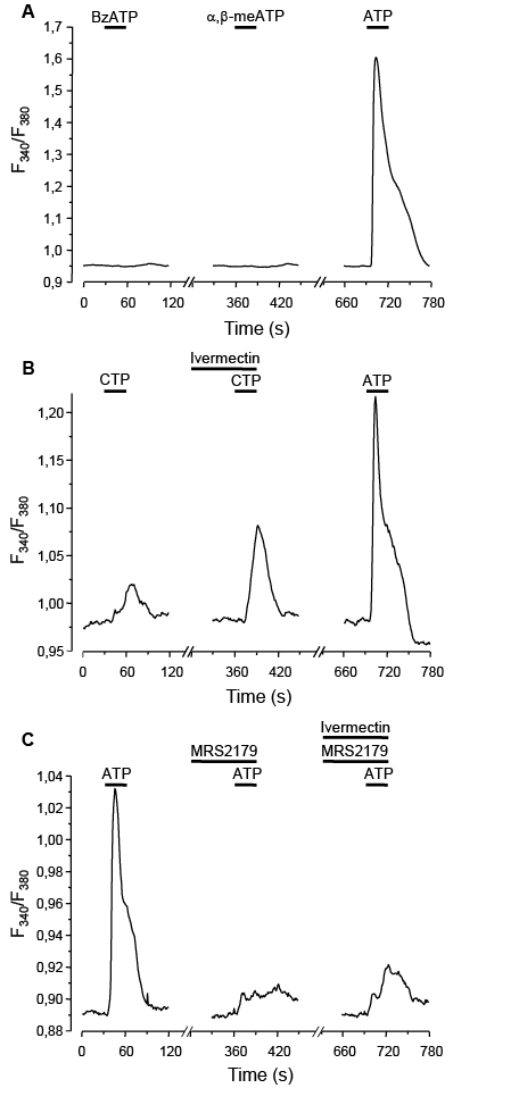

Figure 5. Intracellular calcium increments evoked by different purinergic agonists and effect of the P2X4 potentiator ivermectin in the SK-N-MC cells. (**A**) Effect of 100 μM BzATP or 100 μM α,β-meATP on the intracellular Ca^{2+} concentration in the SK-N-MC cells. At the end of the experiments, cells were challenged with 100 μM ATP to test their

functionality. Traces represent the mean of the responses obtained in 108 individual cells. (**B**) SK-N-MC cells were stimulated with 300 μM CTP both in the absence and in the presence of the P2X4 positive modulator ivermectin (5 μM). At the end of the experiments, cells were challenged with 100 μM ATP to test their functionality. Traces represent the mean of the responses obtained in 135 individual cells that showed responses to CTP. (**C**) SK-N-MC cells were stimulated with 100 μM ATP, both in the absence and in the presence of the $P2Y_1$ antagonist MRS2179 (10 μM), to selectively analyze the MRS2179-resistant component of the ATP response. Afterwards, cells were challenged with 100 μM ATP in the presence of MRS2179 (10 μM) and ivermectin (5 μM), in order to analyze the effect of the positive modulator of the P2X4 receptor on the MRS2179-resistant component of the ATP response. Traces are the mean of the responses measured in 140 individual cells. Horizontal bars above traces indicate duration of drug applications in all cases.

4. DISCUSSION

The limited biological material and the cellular heterogeneity of primary neuronal cultures frequently represent a disadvantage for the *in vitro* study of the nucleotide mediated signalling in the nervous system. The use of homogeneous cultured neuroblastoma cell lines that constitutively express P2 receptors allows avoiding these inconveniences, so these lines could constitute a reliable and convenient model with which analyze the signal transduction pathways and intracellular events associated with the activation of nucleotide receptors in neural tissues.

In the present study we have investigated the presence of functional P2 receptors in human neuroblastoma SK-N-MC cells by a combination of immunological and calcium measurement experiments. Immunological assays showed the expression of $P2Y_1$, $P2Y_6$, $P2Y_{11}$ and $P2Y_{13}$ receptors in the SK-N-MC cell line. P2X1, P2X4, P2X5, P2X6 and P2X7 subunits can also be detected in the SK-N-MC cells by means of immunocytochemical and western blot assays.

Despite the abundant expression of P2 receptors in the SK-N-MC cells only a few range of P2X or P2Y agonists are able to elicit calcium responses. A similar feature can be also observed in other neuroblastoma cell types. For instance, murine N2a cells express a wide range of P2X subunits, including P2X1, P2X3, P2X4 and P2X7. However, of these, the P2X7 receptor was the only functional receptor (6).

The finding that ADP, 2-MeSADP and ADPβS induce calcium increases that are inhibited by the subtype-selective $P2Y_1$-antagonist MRS2179 (36, 37), strongly support the presence of a functional $P2Y_1$ receptor in the SK-N-MC cell line. ADP and its derivatives also activate the $P2Y_{13}$ receptor (36, 38, 39), which can be immunologically detected in SK-N-MC cells. However if the calcium transient induced by these compounds were, at least in part, due to their interaction with a $P2Y_{13}$ receptor, a residual response should be observed in the presence of MRS2179 that, at concentrations up to 100 μM, had no significant effect on the $P2Y_{13}$ receptor (36, 38). This is not the case: responses to ADP and ADP derivatives are almost completely blocked by MRS2179, thus indicating that these agonists increase the intracellular calcium concentration due to their exclusive interaction with a $P2Y_1$ receptor in the SK-N-MC cells. On the other hand, the lack of effect of UDP, a selective agonist at the pyrimidinergic $P2Y_6$ receptor (36, 37), precludes the presence of functional $P2Y_6$ receptors in the SK-N-MC cells, in spite of our data showing their robust expression.

Regarding ATP, this nucleotide was also able to induce calcium transients in the SK-N-MC cells. The partial inhibition that both the specific $P2Y_1$ antagonists MRS2179 and the P2X antagonist TNP-ATP (40) exert on the ATP responses indicates that this nucleotide is activating at least two different receptors in the SK-N-MC cells: the previously mentioned PY_1 receptor, which is blocked by MRS2179, and a P2X receptor, inhibited by TNP-ATP. $P2Y_{11}$ receptor, which is sensitive to ATP (36, 37), can be also detected at the protein level in the SK-N-MC cells. However, if ATP were activating also this receptor, a residual response to the agonist should be observed in the combined presence of MRS2179 and TNP-ATP. This is not the case: ATP responses in SK-N-MC cells are completely blocked by means of a combination of MRS2179 and TNP-ATP. Additionally, the lack of effect of BzATP and UTP, both potent agonists at the $P2Y_{11}$ receptor (36, 41), also precludes the presence of functional $P2Y_{11}$ receptors in the SK-N-MC cell line.

We tried to establish the identity of the P2X receptor that is responsible for the MRS2179-resistant component of the ATP response. SK-N-MC cells did not respond to α,β-meATP or BzATP but increased their $[Ca^{2+}]_i$ when challenged with CTP. Although P2X1 and P2X6 subunits can be immunodetected in the SK-N-MC cells, the presence of functional P2X1 or P2X6 channels can be ruled out due the lack of action of α,β-meATP, a potent agonist of both receptors (42-44). α,β-meATP is a weak or inactive agonist at the P2X5 and P2X7 receptors but, if these channels were functional in the SK-N-MC cells, responses to BzATP should be obtained, as both receptors are activated by this nucleotide analog (42, 45, 46). The pattern of agonist activity observed in the SK-N-MC cells is, however, consistent with the presence of a functional P2X4 receptor. P2X4 channels have been proved to be nearly insensitive to methylene-substituted ATP analogs, whereas they can be activated by CTP, this compound being a less potent agonist than ATP (42, 47). An unusual property of the P2X4 receptor that differentiates it from all other homomeric P2X channels is its potentiation by ivermectin. This antiparasitic agent is a positive allosteric modulator that specifically augments the response of P2X4 receptor to its agonists (34, 35). SK-N-MC cells showed responses to CTP that increased in the presence of ivermectin, this being an additional evidence favouring the presence of a functional P2X4 receptor in the SK-N-MC cell line. Moreover, the residual responses to ATP in the presence of MRS2179 were also potentiated by ivermectin, which could indicate that the P2X4 receptor is responsible for the MRS2179-resistant component of the ATP response in the SK-N-MC cells.

As SK-N-MC cells express different P2X subunits, the possible formation of heteromeric assemblies, in which P2X4 subunits were present, should be taken into consideration. Three different P2X4-containing heteromeric channels have been described so far: P2X1/4, P2X4/6 and P2X4/7 receptors (48-50). P2X4/7 heteromeric receptor shares some of the pharmacological properties of the P2X receptor in the SK-N-MC cells, as it is potentiated by ivermectin (48). However there is a clear difference between both receptors: heteromeric P2X4/7 receptor is activated by BzATP (48) whereas the P2X receptor in SK-N-MC cells has been proved to be insensitive to this nucleotide analog. Regarding P2X1/4 and P2X4/6 receptors, the phenotype of such heteromers differs in some aspects from that of P2X4. For example, heteromeric P2X1/4 and P2X4/6 receptors are activated by low concentrations of α,β-meATP, whereas homomeric P2X4 receptors are relatively insensitive to this substance (49, 50). As P2X receptors in SK-N-MC cells appear to be unaffected by α,β-me-ATP, it seems more probable that they were formed by a homomeric combination of P2X4 subunits rather than a heteromeric combination of P2X4 with the P2X1 or the P2X6 subunits. However, as SK-N-MC cells also express some additional P2X subunit, such as P2X5, a heteromeric association between this subunit and the P2X4 one can not be ruled out.

Taken together, these pharmacological data likely indicate the presence of two functional P2 receptors in the human neuroblastoma SK-M-MC cell line: one metabotropic, $P2Y_1$, and the other ionotropic, P2X4 (or a heteromeric channel including the P2X4 subunit). Responses to ADP and its derivatives will be mediated trough the activation of the $P2Y_1$ receptor, although responses to ATP will be due to the interaction of such nucleotide with both the $P2Y_1$ and the P2X4 receptors. CTP, on their hand, will only activate the ionotropic P2X4 receptor.

P2X4 receptor, which is expressed in several regions of the brain and spinal cord (51, 52), has received considerable recent attention due to its emerging role in the modulation of chronic inflammatory and neurophatic pain (53-55). There are also evidences suggesting that P2X4 receptors could be involved in the mechanism sustaining neuronal cell death caused by oxygen/glucose deprivation (56, 57). More recently, a role of the P2X4 receptor in the long term potentiation in the hippocampus has been described (58).

Regarding $P2Y_1$, immunohistochemical studies showed an abundant and widespread distribution of this receptor throughout the human brain, suggesting its involvement in a number of important functions (59). In post-mortem brain sections from persons with Alzheimer's disease, the $P2Y_1$-like immunoreactivity in the hippocampus and entorhinal cortex was localized to neurofibrillary tangles, neuritic plaques and neuropil threads, which are characteristic Alzheimer's structures (60). On the other hand, it is now recognized that interactions between G protein-coupled receptors can take place through the formation of oligomers, a process commonly referred to as receptor dimerization. There is evidence that $P2Y_1$ receptors can form both homodimers and heteromeric complexes with the $P2Y_{11}$ or A1 receptors (61, 62). Such kinds of interaction will contribute to add complexity to the purinergic signalling

5. CONCLUSIONS

In summary, $P2Y_1$ and P2X4 receptors are widespread in the CNS where they can participate in a number of different and important functions. As these receptors are constitutively expressed and functional in SK-N-MC cells, such cells can constitute a useful model with which analyze several aspects of the physiology of both nucleotide receptors. These include their regulation, associated intracellular signalling pathways, oligomerization, cross-talk with other neurotransmitter systems and putative implication in neurological or neurodegenerative disorders.

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7. ABBREVIATIONS LIST

2-MeSADP, 2-methylthyo-adenosine-5'-diphosphate; α,β-meATP, α,β-methylene-adenosine 5'-triphosphate; ADPβS, adenosine-5'-0-(2-thiodiphosphate); APP, amyloid precursor protein; BSA, bovine serum albumin; BzATP, 2'(3')-O-(4-benzoylbenzoyl)adenosine 5'-triphosphate; ERK1/2, extracellular signal-regulated protein kinase 1/2; Fura-2 AM, fura-2 acetoxymethyl ester; MAPKs, mitogen-activated protein kinases; MRS2179, N^6-methyl-2'-deoxyadenosine-3',5'-bisphosphate; PBS, phosphate-buffered saline; PKC, protein kinase C; PPADS, pyridoxalphosphate-6-azophenyl-2',4'-disulphonic acid; TNP-ATP, 2',3'-*O*-(2,4,6-trinitrophenyl)adenosine 5'-triphosphate.

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